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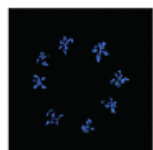
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Rhamnogalacturonan-II cross-linking of plant pectins via boron bridges occurs during polysaccharide synthesis and/or secretion

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Rhamnogalacturonan-II (RG-II), a domain of plant cell wall pectins, is able to cross-link with other RG-II domains through borate diester bridges. Although it is known to affect mechanical properties of the cell wall, the biochemical requirements and lifecycle of this cross-linking remain unclear. We developed a PAGE methodology to allow separation of monomeric and dimeric RG-II and used this to study the dynamics of cross-linking in vitro and in vivo. *Rosa* cells grown in medium with no added boron contained no RG-II dimers, although these re-appeared after addition of boron to the medium. However, other *Rosa* cultures which were unable to synthesize new polysaccharides did not show dimer formation. We conclude that RG-II normally becomes cross-linked intraplastically or during secretion, but not post-secretion.

Boron (B), available in soil as soluble boric acid, is an agriculturally important element for which plants have an absolute requirement.^{1,2} There is a narrow range of optimal concentrations and B deficiency or excess in soil are both problematic, with a range of symptoms such as shortened roots and stems, death of growing-points or roughening of the epidermis.^{3,4,5,6}

Early investigations into the effects of B deficiency revealed strong effects on the mechanical properties of plants, with tissues often feeling 'brittle',^{1,7} suggesting an important role for B in the structure of plant cell walls. Further studies found a correlation with pectin content,⁸ and it

was hypothesized⁷ that pectic apiose residues, found in the rhamnogalacturonan-II (RG-II) component of pectin,^{9,10,11,12,13} bind B, resulting in the ability to form RG-II dimers cross-linked through B (Fig. 1A).

We decided to investigate the formation of RG-II–(B[−])–RG-II bridges and developed a new technique for the separation of RG-II monomers and dimers.¹⁴ Using standard gel electrophoresis equipment, we successfully separated these two compounds (which have similar charge:mass ratio) thanks to their difference in size (~5 and 10 kDa respectively), as is performed for protein SDS–PAGE or oligosaccharide PACE (Fig. 1B).¹⁵ This technique also allows fluorographic visualization of radioactive products, which were produced by radiolabelling RG-II preparations with NaB³H₄. We demonstrated how this technique allows the rapid monitoring of cross-linking by successfully monomerising dimers of RG-II in vitro (by lowering pH with the addition of 0.1 M HCl) and dimerizing monomers (by adding 0.1–1.0 mM H₃BO₃, a process which was promoted by the addition of Pb²⁺).¹⁶

In order to study this cross-linking in vivo, we attempted to grow *Rosa*, *Arabidopsis* and *Spinacia* cell-suspension cultures with reduced H₃BO₃ concentrations in their respective media. The *Arabidopsis* and *Spinacia* cells did not survive in these conditions, but the *Rosa* cells continued to grow (and have been growing for > 2 y). After 8 wk in media with no added boron, only monomeric RG-II was detectable through gel electrophoresis. Re-addition

Keywords: rhamnogalacturonan-II, gel electrophoresis, pectin, boron, radiolabelling, cross-linking, cell wall, *Rosa* sp., *Arabidopsis thaliana*

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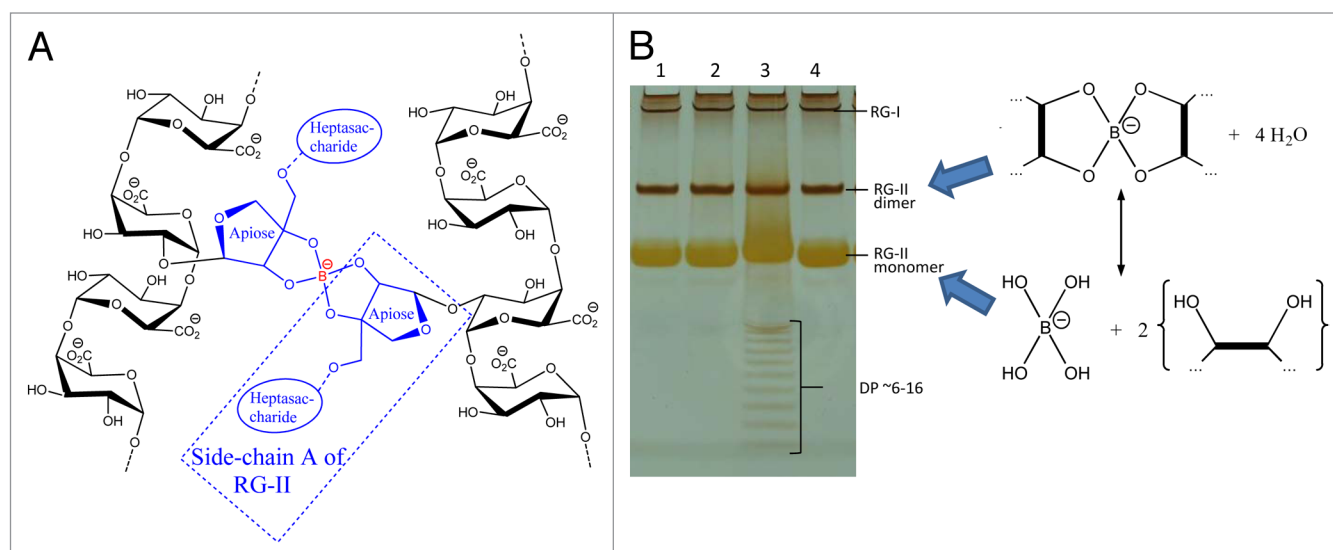


Figure 1. Boron-bridging of RG-II and separation by gel electrophoresis. (A) Schematic representation of boron-bridging of two pectin molecules (B) Gel electrophoresis separation of monomeric and dimeric RG-II. Sample 3 shows separation of oligomers with DP ~6–16 (hexasaccharide to hexadecasaccharide) resulting from the incomplete digestion of homogalacturonan.

of H_3BO_3 to the medium at the routine concentration (3.3 μM) led to the formation of small amounts of dimeric RG-II after 1 h, with the proportion increasing over 24 h. Interestingly, the quantities of monomeric RG-II did not decrease, suggesting that pre-formed monomeric domains were unable to dimerize and only newly formed RG-II formed dimers. To further test this hypothesis, we applied treatments (carbon starvation, respiratory inhibitors, anaerobiosis, freezing or boiling) to *Rosa* cells which decrease or prevent *de-novo* polysaccharide synthesis before re-supplying H_3BO_3 . In all cases, negligible RG-II dimer formation was observed. Importantly, our data also showed that although boron bridges are important for the development of a cell, they are not essential for retaining pectin in the cell wall. Washing the zero-boron cells with Na_2CO_3 did not remove the pectins demonstrating they were an integral component of the cell wall. This is likely to be due to cross-linking with other

cell wall components, possibly through Ca^{2+} -bridges or glycosidic bonds.^{17,18} The mechanism by which Pb^{2+} promotes cross-linking is not understood¹⁶ and it is hypothesized that there may be other substances carrying a similar function in vivo. These could include enzymes, boron carriers or cationic RG-II chaperones, any of which may promote dimerization. We added monomeric RG-II and H_3BO_3 to spent cell culture medium and monitored dimerization by gel electrophoresis. Additionally, we used low concentrations of radiolabelled [^3H]RG-II in case excess amounts of RG-II may be unfavorable to dimer formation. In neither case was dimer formation observed, supporting our hypothesis that cross-linking occurs intraprotoplasmically or during secretion. We also conducted *in-vitro* experiments to investigate the basis of B toxicity. Increasing B concentrations up to 2000-fold did not compromise the dimerization in vitro, but it would be of scientific interest to further investigate the

toxicity problems by supplying excess B in vivo.

The complete 'career' of an RG-II domain is still unknown, and boron cross-linking could occur at several different stages, such as during synthesis in the Golgi bodies, during section into the cell wall, or very soon after secretion into the wall. Further studies into the possibility of Pb^{2+} 'mimics', RG-II chaperones, or boron 'donor substrates' are required to understand this mechanism better, which may provide an insight into why B is such an essential element for plant cell wall development and why excess B is phytotoxic.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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